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Immunohistochemical analysis of sympathetic involvement in the SOD1-G93A transgenic mouse model of amyotrophic lateral sclerosis

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Abstract
Amyotrophic lateral sclerosis (ALS) is a progressive and fatal disorder caused by degeneration of motor neurons in the cerebral cortex, brainstem and spinal cord. Several clinical reports indicated sympathetic and parasympathetic dysfunction in ALS patients. In addition, we have recently reported elevated heart rate and blood pressure in transgenic (TG) mice carrying the SOD1 mutant form of the human SOD1 transgene (SOD1-G93A) even prior to the appearance of motor symptoms. In order to further elucidate the mechanisms underlying autonomic impairment in ALS we performed an immunohistochemical study of the intermediolateral nucleus (IML) column neurons (T2 – L2), superior cervical ganglia (SCG) and adrenal glands (AG) in TG and littermate wild-type (WT) mice at the age of 75 – 80 days. IML column neurons sections were stained with antibodies against choline acetyltransferase (ChAT), SOD1, ubiquitin and SMI31. SCG and AG sections were stained with antibodies against ChAT and tyrosine hydroxylase (TH). ChAT is predominantly located at preganglionic nerve terminals that innervate the AG, while TH is located in sympathetic neurons. Results showed that ChAT, SOD1 and ubiquitin expressions in IML column were significantly lower in the SOD1-G93A group compared to WT (p < 0.0001, p = 0.0042 and p < 0.0001, respectively). SMI31 measurements did not reveal any statistical differences between the two groups (p = 0.6187). TH expression in AG revealed a 24% decrease in the SOD1-G93A group compared to WT (p < 0.0001), while ChAT expression in the SCG was reduced by 28% (p < 0.0001). No significant differences were found for TH in SCG or for ChAT in AG. In conclusion, these results are consistent with preganglionic sympathetic denervation as a potential contributor to the abnormal sympathetic regulation in ALS.

Key words: Amyotrophic lateral sclerosis, SOD1-G93A, superior cervical ganglion, adrenal gland, tyrosine hydroxylase, choline acetyltransferase, autonomic function, transgenic animals

Introduction
Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease. Classically, ALS is defined as a disease with selective progressive degeneration of upper and lower motor neurons eventually leading to paralysis and ultimately death (1). It is increasingly recognized, however, that non-motor manifestations may also occur, including autonomic dysfunction (2). We have recently reported that transgenic (TG) mice carrying a SOD1-G93A mutation, (SOD1-G93A), which leads to motoneuron degeneration, have a higher heart rate (HR) at rest and following stress, and higher blood pressure (BP) than wild-type (WT) littermates, even prior to the appearance of motor symptoms, while at the advanced stages HR and BP gradually fell in these SOD1-G93A mice (3,4).

Sympathetic fibres to the head structures originate from neurons in the intermediolateral horn (IML) of the thoracic spinal cord (SC), at the level of T1 and T2, and form synapses in the superior cervical ganglion (SCG). Postsynaptic fibres innervate cranial blood vessels, sweat glands and smooth muscles (5). The adrenal gland (AG) is innervated exclusively by the sympathetic nervous system (6). Preganglionic fibres originate in the IML of the lower
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At age 75 – 80 days, under deep anaesthesia, animals following their removal, SC, AG and SCG were kept in phosphate buffered saline (PBS). Following their removal, SC, AG and SCG were kept in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Formalin-fixed (PFA 4%), paraffin embedded, 6-μm-thick serial coronal tissue from SC sections were stained with haematoxylin and eosin (H & E) and luxol fast blue (LFB) in order to identify the IML at the levels of the thoracic and lumbar SC segments (T2–L2). All specific immunohistochemical analyses were then performed at serial sections adjacent to those where the IML was identified. Together with IML, the correspondent spinal cord section was analysed as a whole.

**Methods**

The mice used in this study were inbred C57BL/6 mice obtained from the Tel-Aviv University animal breeding centre and TG mice of strain B6SJL-TgN(SOD1-G93A)1Gur/J (Jackson Laboratories, Bar Harbor, Maine). Progeny for experimental analysis were obtained by breeding male SOD1-G93A and female C57BL/6 WT mice. Offspring were genotyped by PCR of tail clips removed after completion of the experiments, and histochemical studies were performed blinded to the mouse genotype.

The study comprised 11 female mice aged 75 – 120 days. Five SOD1-G93A and six WT mice were examined. They were maintained at the Tel-Aviv University Medical School animal facility and cared for in accordance with the guidelines published by the National Institutes of Health (12). At the day of the sacrifice the mice were examined clinically; there was no trembling/dance with the guidelines published by the National Medical School animal facility and cared for in accordance with the guidelines published by the National Institutes of Health (12). At the day of the sacrifice the mice were examined clinically; there was no trembling/dance with the guidelines published by the National Medical School animal facility and cared for in accordance with the guidelines published by the National Institutes of Health (12). At the day of the sacrifice the mice were examined clinically; there was no trembling/dance with the guidelines published by the National Medical School animal facility and cared for in accordance with the guidelines published by the National Institutes of Health (12). At the day of the sacrifice the mice were examined clinically; there was no trembling/dance with the guidelines published by the National Medical School animal facility and cared for in accordance with the guidelines published by the National Institutes of Health (12).
(Chemicon, AP106B) as secondary antibody was used and sections were then treated with avidin-biotin-peroxidase buffer aqueous solution (Sigma). 3-3-diaminobenzidin (DAB) (Sigma) was used as chromogen. Counterstaining was performed with haematoxylin to mark nuclei.

In order to identify the localization of ChAT in SCG, double immunofluorescence together with specific marker for SMI31, which recognizes phosphorylated neurofilament H protein (pNF-H), components of the axons, was performed. Briefly, frozen sections were air dried for 1 h and then hydrated in distilled water. The sections were blocked in FBS 10% for 30 min, then treated with primary antibodies against ChAT and SMI31, followed by incubation with secondary antibodies, donkey anti-goat (Alexa Fluor 568) and chicken anti-mouse (Alexa Fluor 488). The sections were mounted with 4,6-diamidino-2-phenylindol (DAPI) staining (Invitrogen).

Microscopic evaluation
Sections were examined under a Zeiss Axioplan-2 optical microscope with the aid of a CCD camera (Nikon DS-5M) for observations of the slides by two independent observers. Measurements were performed with Image J software 1.43u. On average, 6-12 high and low power visual fields were examined from four sections per animal (five SOD1-G93A and six WT) under a magnification of x20 or x40. Measurements for all but SMI31 expression were performed in the IML of the SC bilaterally in five randomly selected sections from T2-L2 and the results presented as total number of positive neurons per tissue square millimetre along the IML column. SMI31, in particular, was measured as integrated density of each individual neuron along the IML column at the correspondent levels of SC.

In order to evaluate the TH and ChAT staining intensity, in SCG and AG, captured optical fields were converted into 8-bit images and the grey level integrated density was measured.

Statistical analysis
Analysis of the data was performed using the GraphPad Prism 5.0 software. Normality was tested using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Parametric data were analysed using unpaired t-test. Non-parametric data were analysed using the Mann-Whitney test. All determinations were made with a 95% confidence interval and were considered significant at the $p < 0.05$ level.

Results

**Intermediolateral nucleus column**

H & E and LFB staining of the IML column revealed loss of IML neurons and presence of vacuoles in the neuropil, although not intracellularly, among SOD1-G93A mice compared to WT (Figure 1). However, many cytoplasmic vacuoles and aggregates were identified in spinal motor neurons of the anterior horn stained with SOD1 and ubiquitin, respectively (Figure 2). ChAT-positive neurons in the IML column exhibited a statistically significant decreased number in the TG compared to WT group ($p < 0.0001$, Figure 3). Similar results were obtained for SOD1-positive cells ($p = 0.0042$, Figure 3). Further analysis of ubiquitin expression in the IML column revealed also a statistically significant reduction in the SOD1-G93A compared to the WT groups of animals ($p < 0.0001$, Figure 4). SMI31 cellular expression measurements did not reveal any statistical differences between the two groups ($p = 0.6187$, Figure 4).

**Superior cervical ganglia and adrenal glands**

Under light microscopy no evidence of necrotic or apoptotic cell death was identified (data not shown). ChAT expression in SCG, visible as a reduction in DAB staining (i.e. decrease in brown staining), was reduced by 28% ($p < 0.0001$) compared to WT mice ($p < 0.0001$, Figure 5a,b; C,D). There was no difference of TH expression in SCG ($p = 0.5941$, Figure 4a,b; A,B) between the two groups of animals. ChAT was expressed both in neural cell bodies and axons. In particular, WT group exhibited strong expression of ChAT and colocalization with SMI31 in axons and around large neurons. However, in the SOD1-G93A group expression of ChAT in axons was minor and absent around large neurons (Figure 6).

Analysis of TH expression in AG demonstrated a significant decrease in the SOD1-G93A group compared to WT ($p < 0.0001$, Figure 7a,b; A,B) and was evident as a decrease in DAB staining. Quantitatively, there was a 24% reduction in TH expression among the SOD1-G93A mice with respect to their littermates. There was no difference of ChAT expression in AG ($p = 0.1144$, Figure 7a,b; C,D) between the two groups of animals.

Discussion

The aim of the present study was to evaluate the autonomic nervous system, central and peripheral, in SOD1-G93A mice by means of histology and immunohistochemistry. Such a study might shed light on at least some of the clinical sympathetic disturbances observed in patients with ALS. The IML column neurons, the SCG and the AG were examined in SOD1-G93A and WT mice. We examined 11 female mice before the onset of motor symptoms (which is evident at approximately 90–120 days of age (11)). IML columns neurons sections were stained with antibodies against ChAT, SOD1, ubiquitin and SMI31; and the SCG and AG were stained with antibodies against ChAT and TH. We found
that ChAT, SOD1 and ubiquitin expressions in IML column were significantly lower in the SOD1-G93A group compared to WT. As previously reported (13), no cytoplasmic aggregates inside the neurons in the IML column or Bunina bodies among SOD1 or ubiquitin-positive cells were noticed. Nevertheless,

Figure 1. IML column (marked within the dotted area) in WT group at the level T8, as presented with H-E (A) and LFB (C). Loss of IML column neurons and presence of vacuoles in Tg group as indicated by H & E (B) and LFB (D). Scale bars: 100 μm.

Figure 2. SOD1 (A, B) and ubiquitin (D, E) expression in WT (A, C) and Tg (B, D) group in motor neurons of spinal cord anterior horn. Many vacuoles in the neuropil and also inside the cytoplasm of neurons (black arrows, B) and ubiquitin-positive aggregates (black arrows, D) were identified in Tg, whereas the WT group appeared normal. Scale bars: 100 μm.
accumulation of neurofilaments in the cell body and proximal axon of motor neurons is the most characteristic pathological finding in ALS (16,17). Moreover, phosphorylated neurofilament heavy subunit (pNF-H) in peripheral blood and CSF has been proposed as a potential biomarker in ALS (18). However, with regard to pathology, our finding coincides with many cytoplasmatic vacuoles (SOD1) in motor neurons of the anterior horn were identified, possibly derived from degenerating mitochondria (4,15). Interestingly, there was no statistical difference in SMI31 (pNF-H) expression between the two groups ($p = 0.6187$), a finding that seems controversial, bearing in mind that the general concept that abnormal accumulation of neurofilaments in the cell body and proximal axon of motor neurons is the most characteristic pathological finding in ALS (16,17). Moreover, phosphorylated neurofilament heavy subunit (pNF-H) in peripheral blood and CSF has been proposed as a potential biomarker in ALS (18). However, with regard to pathology, our finding coincides with

Figure 3. ChAT (A, B) and SOD1 (D, E) expression in WT (A, D) and Tg (B, E) group in IML column. Graphs indicate the total number of ChAT (C) and SOD1 (F)-positive neurons in IML. Scale bars: 100 μm.
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neurotransmitters acetylcholine and norepinephrine, respectively (8,9), and since these neurotransmitters affect, among others, HR and BP (5), the results of this study may be relevant to our previous clinical findings in this animal model — increased HR and elevated BP (3,4).

Pathological studies have reported the involvement of the preganglionic IML column neurons in human ALS (20,21) in both sporadic (13,22–25) and familial (26) cases, although others (27) found the IML to be apparently normal. Itoh et al. (24) concluded that a degenerative process similar to that seen in motor neurons might take place in the IML.

Figure 4. Ubiquitin (A, B) and SMI31 (D, E) expression in wild-type (WT) (A, D) and transgenic (Tg) (B, E) group in IML column. Graphs indicate the total number of ubiquitin-positive neurons (C) and SMI 31 integrated density (In. Den.) (F) at the area of intermediolateral nucleus (IML). Scale bars: 100 μm.

A recently published analysis of pNF-H (SMI31 antibody) in neurons where similar level of expression both in control and mtSOD1-ALS patients was detected and therefore was not considered to be pathological. On the contrary, large SMI31-positive neuroaxonal spheroids were detected in all non-mtSOD1 variants of ALS in the same study (19). Moreover, TH expression in AG and ChAT expression in SCG were both decreased in the SOD1-G93A compared to the WT group \(p < 0.0001\). On the other hand, TH expression in the SCG and that of ChAT in AG were not affected. Since ChAT and TH are enzymes that catalyse the synthesis of the neurotransmitters acetylcholine and norepinephrine, respectively (8,9), and since these neurotransmitters affect, among others, HR and BP (5), the results of this study may be relevant to our previous clinical findings in this animal model — increased HR and elevated BP (3,4).
SCG is known to contain ChAT-positive fibres, presumably originating in the cervical IML (28,29). Following surgical preganglionic denervation of the SCG, all ChAT-positive fibres disappear (29). The downward progression of the IML neuronal loss (13) may explain our findings that ChAT decreases in SCG although not in AG, which is innervated by lower thoracic segments (6,7). The mice examined by us were at a relatively young age (around 11 weeks) and loss in ChAT expression in AG may become evident at later stages.

Although we could not find any alteration of ChAT in the AG, we found, unexpectedly, decreased TH activity. This is reminiscent of findings in column of ALS patients. Takahashi et al. (13) reported that the loss of IML neurons starts from the upper SC and extends to the lower thoracic segment (13). Oey et al. referred to the involvement of this column as the cause of the enhanced sympathetic activity and the absence of sympathetic skin response in ALS patients (20). They postulated that a higher muscle sympathetic nerve activity at rest and decreased response to sympathoexcitatory stimuli are caused by changes in the modulation of preganglionic IML column neurons. Our findings in the IML are in accordance with findings in humans (13,24) and point to a degeneration of preganglionic neurons of the IML.
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Figure 6. Fluorescence double immunohistochemistry for SMI 31 (phosphorylated monoclonal antibody) (A, D) and ChAT (B, E), in wild-type (WT) (A, B, merged in C) and transgenic (TG) (D, E, merged in F) mice in superior cervical ganglia. Both neural cell bodies (SMI 31-negative, arrows in A, D) and axons (SMI 31-positive, arrowheads in A, D) were positive for ChAT (correspondent arrows and arrowheads in B, E). ChAT expression in TG animals (D, E, F) was preserved in axons (arrowheads), although not in neural cells (arrows) compared to WT animals (A, B, C, correspondent arrows and arrowheads). Scale bars: 100 μm.

Figure 7. a. Tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) immunoreactivity in adrenal gland (AG) cells in 11-weeks-old SOD1-G93A transgenic (Tg) and WT mice. The vertical bars indicate standard error of the mean (SEM). b. TH expression in AG in WT (A) and transgenic (TG) mice (B) and ChAT expression in AG in WT (C) and TG mice (D). TH expression in the AG was significantly reduced in the SOD1-G93A group compared to WT (two right bars in figure a. *p<0.0001). No significant differences were identified for ChAT expression in AG (two left bars in figure a.). Original magnification x20; upper right insert 40x. Nuclear counterstaining with haematoxylin. Scale bars: 100 μm.
spontaneously hypertensive rats (30), in which, even before the development of hypertension, norepinephrine content in plasma was significantly increased. As postulated, feedback inhibition by the elevated norepinephrine plasma levels may be responsible for this reduction. In the same study (30), down-regulation of TH in the sympathetic ganglia did not occur and the authors speculated that the difference in the density of alpha-2-adrenergic receptors subtype between sympathetic nerve terminals and AG and the difference in norepinephrine affinity for these receptors may explain the different TH response. In sympathetic nerve terminals, 90% of the presynaptic adrenergic receptors are of the alpha-2A subtype and the alpha-2C are less abundant (10%), whereas in the AG only alpha-2C receptors occur (30). Norepinephrine has a higher affinity for the alpha-2C subtype than for alpha-2A receptors. This could possibly explain why we observed a reduction in the activity of TH in the AG but not in the SCG.

Centrally-active drugs also produce characteristic and different patterns of TH activation in the peripheral sympatho-adrenal system (31). D-amphetamine, ET-495 (a dopaminergic agonist) and morphine significantly increased TH activity in the AG but failed to modify enzyme activity in the SCG. In addition, levels of adrenal TH were found to be dependent on the innervation of the AG. Denervation of the AG depressed TH activity by approximately one half (32). Thus, the selective responses of TH activity to various stimuli (31) and the dependency of adrenal TH on adrenal innervation (32) may also explain why TH expression was decreased in the AG but not in the SCG.

The preganglionic sympathetic denervation may result in an inappropriate release of norepinephrine. Druschky et al. (33) showed a significantly reduced presynaptic postganglionic uptake of the norepinephrine analog metaiodobenzylguanidine (MIBG) into nerve terminals of the heart in early stages of ALS, suggesting impaired cardiac sympathetic innervation (33). Chida et al. (34) observed that the plasma levels of norepinephrine and epinephrine were significantly higher in sporadic early-onset ALS patients than in controls. Elevated plasma norepinephrine levels were also noted by others (36,37). The high levels of these neurotransmitters are consistent with the increased HR and BP seen in human ALS and in our experimental animals (2–4), but are not explained by the sympathetic denervation observed by Druschky (33) and by Oey (20) and by us. It may well be that the increased levels of catecholamines may be due to impaired reuptake of circulating norepinephrine (since nerve terminals degenerate) or be analogous to the spasticity or fasciculations seen in human disease.

The main advantage of our study was that it was performed before motor symptoms onset with the examiner blinded to the genotype of the animals. However, we have not examined these mice at multiple time-points throughout disease progression. In addition, the study population was relatively small.

In conclusion, the reduced expression of ChAT in the SCG and of TH in the AG detected in the SOD1-G93A mouse model might reflect degeneration of preganglionic neurons of the IML. Our immunohistochemical findings shed further light on the autonomic impairment in ALS.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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